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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Display of both N- and C-terminal target fusion proteins on the *Aspergillus oryzae* cell surface using a chitin-binding module

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Abstract A novel cell surface display system in *Aspergillus oryzae* was established by using a chitin-binding module (CBM) from *Saccharomyces cerevisiae* as an anchor protein. CBM was fused to the N or C terminus of green fluorescent protein (GFP) and the fusion proteins (GFP-CBM and CBM-GFP) were expressed using *A. oryzae* as a host. Western blotting and fluorescence microscopy analysis showed that both GFP-CBM and CBM-GFP were successfully expressed on the cell surface. In addition, cell surface display of triacylglycerol lipase from *A. oryzae* (tglA), while retaining its activity, was also successfully demonstrated using CBM as an anchor protein. The activity of tglA was significantly higher when tglA was fused to the C terminus than N terminus of CBM. Together, these results show that CBM used as a first anchor protein enables the fusion of both the N and/or C terminus of a target protein.

Keywords Cell surface display · *Aspergillus oryzae* · Chitin-binding module · Triacylglycerol lipase

Introduction

The development of cell surface display systems of heterologous proteins or peptides has recently become an active research area (Boder and Witttrup 1997; Stahl and Uhlen 1997; Kondo and Ueda 2004). By utilizing naturally occurring surface proteins as scaffolds, functional proteins can be displayed on various cell surfaces (Hansson et al. 2001). In these systems, cells displaying functional proteins can be easily separated and have been used in a wide variety of biotechnological applications, such as bioconversion (Tateno et al. 2007; Kotaka et al. 2008), bioremediation of heavy metals (Kambe-Honjoh et al. 2000), oral vaccine development (Raha et al. 2005; Ramasamy et al. 2006), and combinatorial library screening (Georgiou et al. 1997). Cell surface display systems require anchor proteins as a scaffold to maintain fused proteins with a high degree of stability within the cell wall. So far, several cell surface display systems using various anchor proteins have been reported in Gram-negative/positive bacteria, yeasts, and so on (Murai et al. 1997; Lee et al. 2003; Tanino et al. 2004).

Aspergillus oryzae has been commonly used in the manufacture of traditional Japanese fermented food products, such as sake, miso (paste made from soybeans), and soy sauce, and has been guaranteed to be generally recognized as safe (GRAS). Recently, the molecular biology of *Aspergillus* species has been studied extensively, including determination of both the entire genome sequence and the expressed sequence tags (Machida et al. 2005; Akao et al. 2007).

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Furthermore, recombinant *A. oryzae* strains have been developed for the production of heterogeneous and endogenous proteins, because this microorganism secretes large amounts of protein (Christensen et al. 1988; Iwashita 2002; Kitamoto 2002). However, to our knowledge, cell surface display systems in *A. oryzae* have only been developed using a glycosylphosphatidylinositol anchor protein (Adachi et al. 2008). Therefore, to expand the *A. oryzae* cell surface display system, the development of other anchor proteins for display on *A. oryzae* is needed.

The site of the displayed protein and anchor protein fusion (i.e., N or C terminus) is one of the important factors in determining whether the target proteins are displayed without loss of function. In yeast and lactic acid bacteria cell surface display systems, it has been demonstrated that the activity of displayed proteins depends on the fusion site (N or C terminus) of the anchor proteins (Shigechi et al. 2004; Okano et al. 2008). α -Amylase from *Streptococcus bovis* 148 (Satoh et al. 1993) and lipase from *Rhizopus oryzae* show high activity when fused to the C terminus of the anchor protein, but poor activity when fused to the N terminus (Shigechi et al. 2004; Washida et al. 2001). For *A. oryzae*, there is currently no anchor protein that allows fusion of a target protein to either the N or C terminus.

In this study, to expand the utility of *A. oryzae*, we established a novel cell surface display system that enables the target protein to fuse to both N and C termini of the anchor protein. We focused on the chitin-binding module (CBM) from *Saccharomyces cerevisiae*, which has high affinity to chitin (Kuranda and Robbins 1991). *A. oryzae* has a large amount of chitin on its cell surface (Seidl 2008; Higuchi et al. 2009), and therefore CBM should be suitable as an anchor protein which tightly binds to the *A. oryzae* cell wall. Using CBM as an anchor protein, we tested the displaying possibilities of green fluorescent protein (GFP) and triacylglycerol lipase from *A. oryzae* (tglA).

Materials and methods

Strains and media

Escherichia coli NovaBlue (Novagen, Inc., Madison, WI) was used as the cloning host for recombinant DNA manipulations. The bacterium was grown in Luria–Bertani medium (1% tryptone, 0.5% yeast extract, and 0.5% NaCl) containing 0.1 mg/ml of ampicillin. The *A. oryzae* niaD mutant (strain IF4), derived from wild-type *A. oryzae* OSI1031, was used as the expression host for the novel cell surface display system. Czapek–Dox (CD) medium plates (2% glucose, 0.3% NaNO₃ (CD-NO₃), 0.2% KCl, 0.1% KH₂PO₄, 0.05% MgSO₄·7H₂O, and 0.8 M NaCl, pH 6.0) containing 1.5% agar were used as the minimal

medium. The plate was used to select the fungal transformants. GPY medium (3% glucose, 0.2% KCl, 0.1% KH₂PO₄, 0.05% MgSO₄·7H₂O, 1% peptone, and 0.5% yeast extract, pH 6.0) was used for growing the *A. oryzae* IF4 and transformants. All transformants and wild-type *A. oryzae* used for all analyses in this study were cultivated in Sakaguchi flasks (500 ml) containing 100 ml of GPY medium.

Construction of expression vectors and *A. oryzae* transformation

A. oryzae transformation vectors were constructed using the pISI vector (Research Institute, Gekkeikan Sake Co., Kyoto, Japan), which contains the *sodM* promoter and *glbB* terminator from *A. oryzae* (Ishida et al. 2004). Polymerase chain reaction (PCR) amplification of DNA fragments was performed using KOD plus DNA polymerase (Toyobo, Osaka, Japan) according to the manufacturer's protocol.

The pISI-GFP vector (Adachi et al. 2008) containing the *tglA* signal sequence from *A. oryzae* (Toida et al. 2000), the N28 sequence from *R. oryzae* (Hama et al. 2006; Hama et al. 2008), and the *EGFP* gene PCR amplified from pEGFP (Clontech Laboratory, Mountain View, CA) was used as the GFP secreting vector. The GFP anchoring expression vectors were constructed as follows. The DNA fragment encoding CBM was amplified by PCR using the genome from the *S. cerevisiae* W303-IB strain using the following primers, 5'-GCTAATGGAGCGGCCGCATCAGACAGTACAGCTCGTACATTGGCTAAAGA-3' and 5'-CCATAGGATATTTAAATCTAAAAGTAATTGCTTTCCAAATAAGAGAAATT-3' (underlined sequences indicate restriction enzyme sites). The amplified fragment was digested with *NotI* and *SwaI*, and inserted into pISI-GFP-MP1 (Adachi et al. 2008). The resultant plasmid was designated as pISI-GFP-CBM. The DNA fragment encoding the last 15 bases of the N28 sequence, CBM, and the last 15 bases of GFP was amplified from the genome of the *S. cerevisiae* W303-IB strain with the following primers, 5'-AACAGCGCCAAGCGTTGGCCATCAGACAGTACAGCTCGTAC-3' and 5'-GCCCTTGCTCACCATCATA TGAAAGTAATTGCTTTCCAAAT-3'. The amplified fragment was inserted into *SaI*-digested linear pISI-GFP using the In-Fusion enzyme (Takara Bio, Otsu, Japan) according to the manufacturer's procedure. The resultant plasmid was designated as pISI-CBM-GFP.

The *tglA* secreting and anchoring vectors were constructed as follows. The DNA fragment encoding the mature region of *tglA* from *A. oryzae* that was fused with the gene encoding the FLAG peptide tag at the C terminus was amplified from cDNA prepared from *A. oryzae* with the following primers, 5'-ACGCGTCC

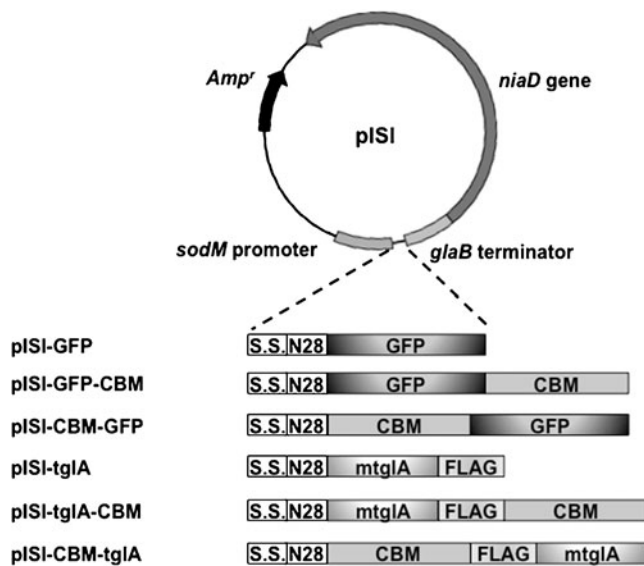


Fig. 1 Expression vectors constructed in this study. *s.s.* secretion signal sequence of *tglA*, *N28* N-terminal 28 amino acid residues of *Rhizopus oryzae* lipase pro-region, *mtgIA* mature region of *tglA*

ACAACGCTCCCCTGAATGAGTTCCTCAGCGCT-3' and 5'-GGTTATTAAATTTACTTGTCATCGTCATCCTTGTAAGTCGTTTCGCAGCCGCAACAGCA

Fig. 3 GFP fluorescence micrographs of *A. oryzae*. *A. oryzae*/GFP-CBM transformant (*a*, *b*, and *c*) *A. oryzae*/CBM-GFP transformant (*d*, *e*, and *f*). *A. oryzae*/GFP transformant (*g*, *h*, and *i*). Enlarged views in the circles in *a*, *d*, and *g* (*b*, *e*, and *h*, respectively). Proteinase K-treated transformants (*c*, *f*, and *i*). Each scale bar represents 50 μ m

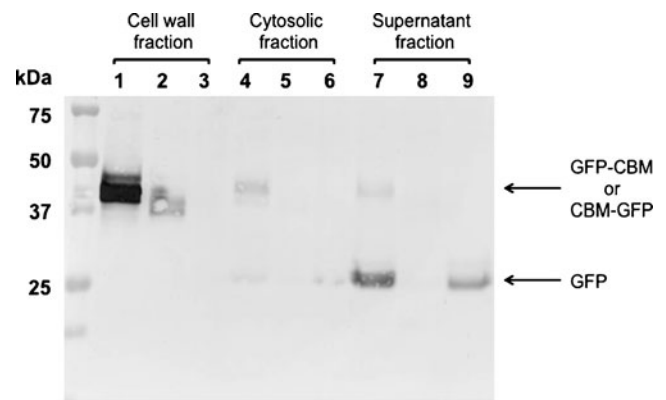
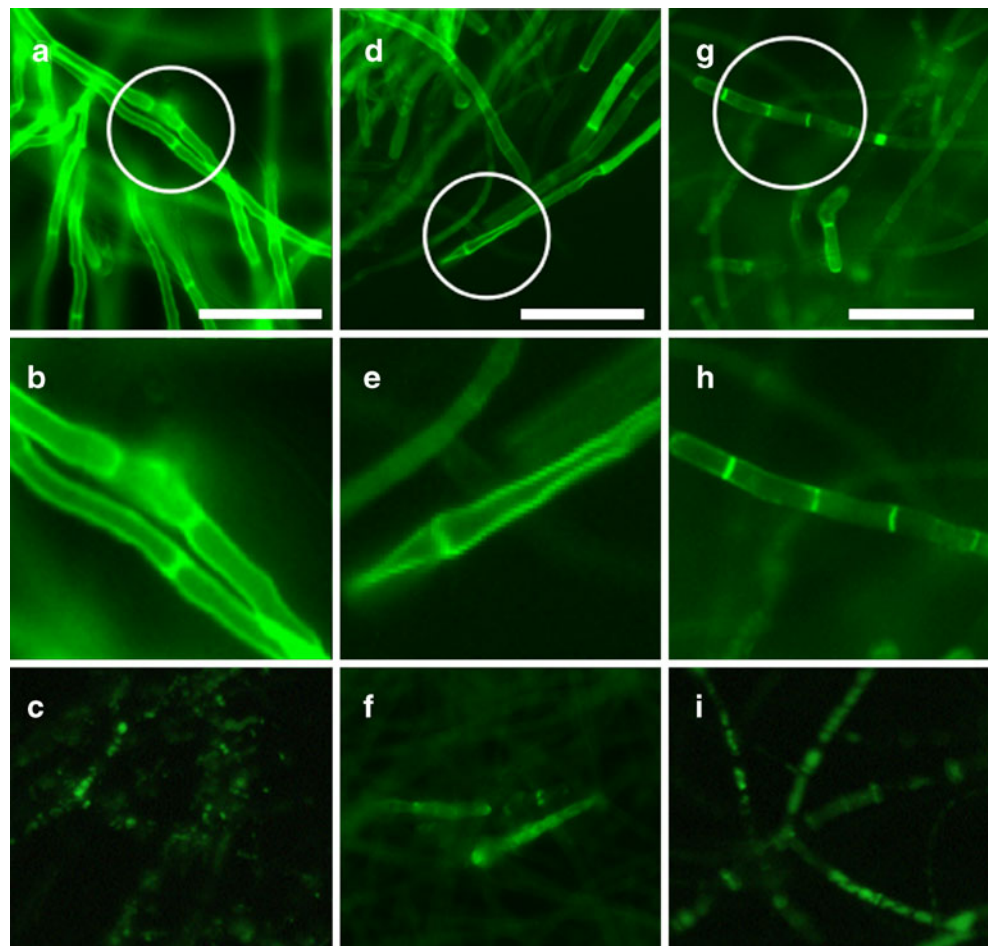


Fig. 2 Western blot analysis of GFP, GFP-CBM, and CBM-GFP fusion proteins from cell wall fractions (lanes 1 to 3), cytosolic fractions (lanes 4 to 6), and supernatant fractions (lanes 7 to 9). Lanes 1, 4, and 7, *A. oryzae*/GFP-CBM transformant; 2, 5, and 8, *A. oryzae*/CBM-GFP transformant; 3, 6, and 9, *A. oryzae*/GFP transformant

A-3' or 5'-GGTTGCGGCCGCCTTGTCATCGTCATCCTTGTAAGTCGTTTCGCAGCCGCAACAGCA A-3'. The amplified fragment was digested with *SalI* and *SwaI*, or *SalI* and *NotI*, respectively, and inserted into pISI-GFP-CBM. The resultant plasmids were designated as pISI-tglA and pISI-tglA-CBM, respectively. The DNA

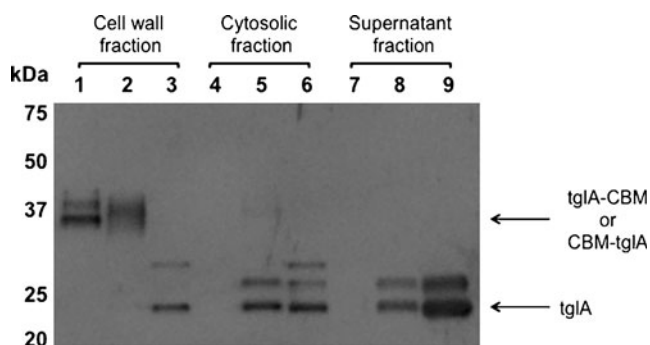


Fig. 4 Western blot analysis of tglA, CBM-tglA, and tglA-CBM fusion proteins from cell wall fractions (lanes 1 to 3), cytosolic fractions (lanes 4 to 6), and supernatant fractions (lanes 7 to 9). Lanes 1, 4, and 7, *A. oryzae*/tglA-CBM transformant; 2, 5, and 8, *A. oryzae*/CBM-tglA transformant; 3, 6 and 9, *A. oryzae*/tglA transformant

fragment encoding the mature region of tglA from *A. oryzae* that was fused with the gene encoding the FLAG peptide tag at the N terminus was amplified from cDNA prepared from *A. oryzae* with the following primers, 5'-GGAATTCATATGGACTACAAGGATGACGATGACAAGAACGCTCCCCTGAATGAGTT-3' and 5'-TCCCATTAAATTTAGTTTCGCAGCCGCAACAG-3'. The amplified fragment was digested with *Nde*I and *Swa*I and inserted into pISI-CBM-GFP. The resultant plasmid was designated as pISI-CBM-tglA. All PCR-amplified DNA fragments were confirmed by DNA sequencing (CEQ 8000, Beckman Coulter). All expression plasmids constructed in this study are summarized in Fig. 1.

Transformation of *A. oryzae* was carried out according to the method described by Gomi et al (1987). The resultant transformants were subcultured on CD-NO₃ medium plates three times to obtain stable expression transformants. The transformants were named *A. oryzae*/GFP, *A. oryzae*/GFP-CBM, *A. oryzae*/CBM-GFP, *A. oryzae*/tglA, *A. oryzae*/tglA-CBM, and *A. oryzae*/CBM-tglA. According to our

previous study, the copy number of the expression vectors integrated into the genome was assumed to be one (Ishida et al. 2000).

Fluorescence microscopy analysis

Wild-type *A. oryzae* and transformants harvested from submerged cultures cultivated for 7 days were washed twice with PBS. GFP fluorescence was detected using a fluorescence microscope (BZ-8000; Keyence Co., Osaka, Japan).

Protease treatment

Recombinant *A. oryzae* harvested from submerged cultures cultivated for 10 days were washed with PBS three times, and the surface-displayed protein was cleaved by incubating the suspension with Proteinase K (final concentration, 30 U/L) for 24 h at 37°C. After proteolysis, *A. oryzae* was washed twice with PBS, followed by gentle centrifugation to remove the protease, and GFP fluorescence was observed using fluorescence microscopy as described above.

Western blot analysis

Cell wall proteins were extracted from wild-type *A. oryzae* and transformants. Briefly, the washed frozen cells were ground with a pestle and mortar, and then resuspended in PBS (15–20 µl/mg dry cell). The cytosolic fraction was separated from cell walls and membranes by centrifugation at 15,000 rpm at 4°C for 10 min. Then the resultant precipitate was washed twice with PBS and subjected to the following analysis as a cell wall fraction.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis was performed using a 12.5% gel. The

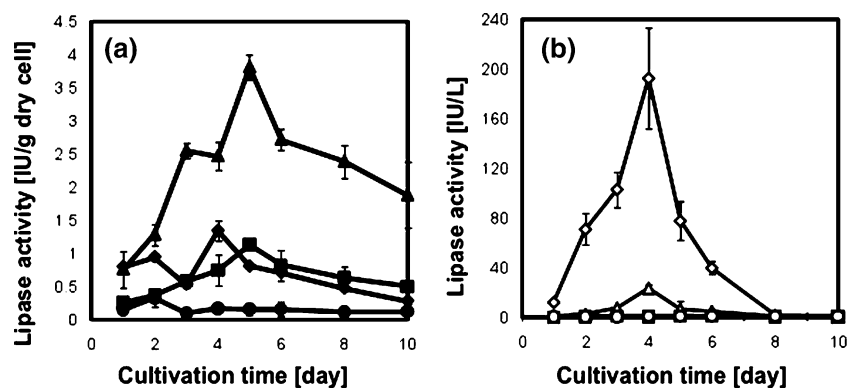


Fig. 5 Time course of lipase activity of the fungus bodies of recombinant *A. oryzae* displaying tglA-CBM and CBM-tglA fusion proteins (a) and that of culture supernatant (b). Symbols represent wild-type (closed circles and open circles), *A. oryzae*/tglA trans-

formant (closed diamonds and open diamonds), *A. oryzae*/tglA-CBM transformant (closed squares and open squares), and *A. oryzae*/CBM-tglA transformant (closed triangles and open triangles)

separated proteins were transferred from the gel to a polyvinylidene difluoride (PVDF) membrane (Atto, Tokyo, Japan) using a HorizBlot AE-6677 (Atto), according to the supplier's instruction. The PVDF membrane was blocked with 5% non-fat milk and incubated with primary mouse anti-GFP IgG (Sigma, St. Louis, MO) or mouse anti-FLAG IgG (Sigma) for 1 h at room temperature. The PVDF membrane was subsequently incubated with a secondary alkaline phosphatase-conjugated mouse anti-mouse IgG antibody (Promega, Madison, WI) and washed twice with TBS-T buffer. For detecting GFP, the PVDF membrane was incubated with a mixture of nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate toluidine salt (Promega), according to the manufacturer's procedure. For detecting tglA, the PVDF membrane was incubated with CDP-star (Applied Biosystems), according to the manufacturer's procedure.

Measurement of lipase activity

Wild-type *A. oryzae* and each of the transformants were cultivated in GPY medium and harvested by filtration with Miracloth (Calbiochem, Darmstadt, Germany). Cells were washed with distilled water and lyophilized. Lyophilized cells were crushed with an SK-Mill (Funakoshi, Tokyo, Japan). Culture supernatants and crushed cells were used for the assay of lipase activity using Lipase Kit S (DS Pharma Biomedical, Osaka, Japan) according to the supplier's protocol, and the resulting values were expressed in international units (IU). One unit of lipase activity was defined as the amount of enzyme catalyzing the formation of 1 mmol of 2,3-dimercaptopropan-1-ol from 2,3-dimercaptopropan-1-ol tributyl ester per min. Triplicate experiments were performed.

Results

Localization of GFP-CBM and CBM-GFP fusion proteins evaluated by western blotting

We constructed GFP-CBM and CBM-GFP fusion protein expression vectors (pISI-GFP-CBM and pISI-CBM-GFP, respectively) as shown in Fig. 1. The GFP secreting vector (pISI-GFP) was used as a control (Adachi et al. 2008). These vectors were transformed into the *A. oryzae* IF4 niaD[−] strain to obtain each transformant. To investigate the expression and localization of GFP-CBM and CBM-GFP proteins, the cell wall fractions of recombinant *A. oryzae* were subjected to western blotting with an anti-GFP monoclonal antibody. The bands corresponding to the GFP-CBM and CBM-GFP fusion proteins were observed

in the cell wall fractions of *A. oryzae*/GFP-CBM and *A. oryzae*/CBM-GFP transformants (Fig. 2, lanes 1 and 2). Although weak bands of GFP-CBM were observed in the cytosolic and supernatant fractions (Fig. 2, lanes 4 and 7), GFP-CBM and CBM-GFP were mainly expressed in the cell wall fraction. For the *A. oryzae*/GFP transformant, GFP was secreted in the supernatant (Fig. 2, lane 9) and did not accumulate in the cell wall fraction (Fig. 2, lane 3). For GFP-CBM, the band corresponding to GFP was also observed in the supernatant fraction (Fig. 2, lane 7), which might have been caused by non-specific proteolysis.

Localization of GFP-CBM and CBM-GFP fusion proteins visualized by fluorescence microscopy

To visualize the localization of GFP-CBM or CBM-GFP, each transformant was directly observed using fluorescence microscopy. Bright green fluorescence was clearly observed on the cell surface of *A. oryzae*/GFP-CBM (Fig. 3a and b) and *A. oryzae*/CBM-GFP (Fig. 3d, e) transformants. In contrast, no green fluorescence signal was detected on the cell surface of the *A. oryzae*/GFP transformant (Fig. 3g, h). These results clearly show that CBM is a useful anchor protein on the *A. oryzae* cell surface allowing function of the target protein to be retained.

To evaluate the accessibility of cell surface displaying GFP, protease treatment was carried out. After proteinase K treatment, green fluorescence on the cell surface of *A. oryzae*/GFP-CBM and *A. oryzae*/CBM-GFP transformants was diminished (Fig. 3c, f), whereas the *A. oryzae*/GFP transformant was scarcely different before and after treatment (Fig. 3h, i). We assumed that we could visualize the produced GFP fused with anchor proteins that were successfully localized on the cell surface.

Evaluation of triacylglycerol lipase-displaying *A. oryzae*

To construct *A. oryzae* transformants displaying triacylglycerol lipase, the pISI-tglA-CBM and pISI-CBM-tglA (Fig. 1) vectors were transformed into the *A. oryzae* IF4 niaD[−] strain. As a control, an *A. oryzae* transformant secreting triacylglycerol lipase was constructed using the pISI-tglA vector. First, the localization of the tglA-CBM and CBM-tglA fusion proteins was investigated by western blot analysis using an anti-FLAG monoclonal antibody (Fig. 4). The bands corresponding to the tglA-CBM and CBM-tglA fusion proteins were observed only in the cell wall fractions from *A. oryzae*/tglA-CBM and *A. oryzae*/CBM-tglA transformants (Fig. 4, lanes 1 and 2). With respect to the cytosolic and supernatant fractions of the *A. oryzae*/CBM-tglA transformant, bands corresponding to tglA were detected (Fig. 4, lanes 5 and 8), which might have been caused by non-specific proteolysis. In the *A.*

oryzae/tgIA transformant, although bands corresponding to tgIA were detected in all of the fractions, it was mainly observed in the supernatant fraction (Fig. 4, lanes 3, 6, and 9).

We then evaluated the time course of lipase activity of the fungus body and culture supernatant (Fig. 5a, b). About 800 mg of dry cells were obtained from the 100 ml of culture medium after 8 days cultivation and there was no significant difference of the dry-cell weight between each transformants (data not shown). There was no activity in the fungus body or culture supernatant of *A. oryzae*, which served as a negative control. As for the culture supernatant, lipase activity (maximal activity was 190 IU/L) was detected only in the *A. oryzae*/tgIA transformant (Fig. 5b). In terms of the fungus body, the lipase activity of the *A. oryzae*/CBM-tgIA transformant (maximal activity was 3.8 IU/mg dry cells) was about 4-fold higher than that of *A. oryzae*/tgIA (maximal activity was 1.1 IU/mg dry cells) and *A. oryzae*/tgIA-CBM (maximal activity was 1.3 IU/mg dry cells) transformants (Fig. 5a). These results indicate that tgIA was successfully displayed on the cell surface by the *A. oryzae*/CBM-tgIA transformant while retaining its enzymatic activity.

Discussion

In this study, to expand the utility of the cell surface display system of *A. oryzae*, we successfully developed a novel cell surface display system using CBM as the anchor protein. CBM has two putative advantages. One is its small size (about 9 kDa) compared to the previously reported anchor protein MP1 (about 25 kDa; Adachi et al. 2008) which may reduce the steric hindrance between anchor protein and target protein, and the other is that a target protein can be fused to either its N or C terminus.

Western blot analysis revealed that both GFP-CBM and CBM-GFP fusion proteins were located in the cell wall fraction (Fig. 2). GFP fluorescence was also clearly detected ubiquitously on the cell surface of *A. oryzae* (Fig. 3a, b, d, e). To our knowledge, CBM is the first anchor protein whose N or C terminus can be fused with a target protein that can be expressed on the cell surface of *A. oryzae* while retaining its activity. The expression level of GFP-CBM is higher compared with that of GFP-MP1 (Adachi et al. 2008), showing one of the advantages of CBM as an anchor protein. The expression level of GFP-CBM is higher compared to that of CBM-GFP, suggesting the importance of the fusion site for the production levels of the fusion protein. In all transformants, the GFP fluorescence localization at the both hyphal tips and septa was caused by the signal sequence, which is consistent with a previous report (Maruyama and Kitamoto 2007). Additionally, the decrease

in cell surface fluorescence after proteinase K treatment clearly shows that GFP is accessible enough for the protease (Fig. 3c, f), which suggests that both GFP-CBM and CBM-GFP were oriented outside the cell wall.

Using CBM as an anchor protein, the lipase tgIA was displayed on the cell surface of *A. oryzae*. Both tgIA-CBM and CBM-tgIA fusion proteins were also successfully located on the cell wall (Fig. 4). Because tgIA activity was detected only in the fungus body, this demonstrates that CBM can immobilize a fused protein tightly on the cell surface in the same manner as the glycosylphosphatidyl inositol anchor protein MP1 (Adachi et al. 2008). However, the lipase activity on the fungus body expressing tgIA-CBM was lower compared to that expressing CBM-tgIA. One possible explanation is that the activity of tgIA-CBM may be inhibited because the location of the catalytic triad of tgIA is on the C-terminal side (Toida et al. 2000). Although introduction of some linker region between target protein and anchor protein may increase the activity of target protein, it is time-consumable and troublesome. These results also suggest that the site of the displayed protein and anchor protein fusion is important for protein display without the loss of function, and that CBM is an appropriate anchor protein due to the availability of both its N and C termini.

In conclusion, we developed a novel cell surface display system for *A. oryzae* using CBM as an anchoring motif. This system is convenient for genetic manipulation because CBM has a small molecular weight and target proteins can be fused to either its N or C terminus. Furthermore, it should be possible to catalyze sequential reactions by combining this CBM-based system with cell surface display using MP1 or other anchor proteins.

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